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Apoptosis in early salivary gland duct morphogenesis and lumen formation

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Abstract

Salivary glands (SG) are essential for the maintenance of oral health by providing lubrication and antimicrobial protection to the mucosal and tooth surfaces. Saliva is modified and delivered to the oral cavity by a complex multifunctional ductal system. During development these ducts form as solid tubes, which undergo cavitation to create lumens. Apoptosis has been suggested to play a role in this cavitation process along with changes in cell polarity. Here we show that apoptosis occurs from the very earliest stages of mouse SG development, much earlier than previously reported. Apoptotic cells were observed in the centre of the first epithelial stalk at early stage E12.5 using both TUNEL staining and Cleaved Caspase-3 immunofluorescence. The presumptive lumen space was highlighted by the co-localisation of a predictive lumen marker, Cytokeratin 7 (K7). At E14.5, as lumens start to form throughout the glands, apoptotic expression decreased while K7 remained positive. *In vitro* inhibition of all caspases in E12.5 and E13.5 SG resulted in wider ducts compared to the controls and a defect in lumen formation. In contrast no such defect in lumen formation was observed at E14.5. Our data indicates that apoptosis is involved during early stages of gland formation (E12.5 onwards) and appears important for shaping the forming ducts.

Introduction

During epithelial organogenesis, lumen formation within an initially solid tube is essential for building functional networks of epithelial tubes in different organs such as mammary and salivary glands (SG) (Kwon and Larsen 2015; Mailleux et al. 2007). It has been postulated that canalisation of SG ducts and other glands is due to clearance of the inner cell population within newly branched epithelial stalks due to apoptosis, programmed cell death. Lumen formation therefore occurs secondarily to branching morphogenesis (Andrew and Ewald 2010; Lubarsky and Krasnow 2003). The role of cell death in SGs, however, has recently been challenged with lumen formation occurring after apoptosis is blocked (Nedvetsky et al. 2014).

As previously described SG development is generally classified into prebud, initial bud, pseudoglandular, canalicular and terminal bud stages, with gland initiation occurring around embryonic day E11 in the mouse (Jaskoll & Melnick 1999). The prebud stage is characterized as a thickening of the oral epithelium, which proliferates towards the underlying mesenchyme and forms an epithelial bud with a narrow stalk region (initial bud stage). The bud then undergoes clefting to create multiple buds that characterize the pseudoglandular stage. The epithelial stalks then undergo canalisation, whilst ductal and acinar cells then differentiate to achieve a mature gland (Jaskoll and Melnick 1999). This pattern of development is very similar to the stages observed during human development (Teshima et al. 2011; Lourenço et al. 2008).

To date, lumen formation in SGs has been reported to start from (Jaskoll et al. 2001; Tucker 2007). Apoptotic cells have been observed localised in the developing ducts and active caspase 3, a central mediator of the apoptotic pathway, is expressed in these regions (Jaskoll and Melnick 1999; Jaskoll et al. 2001). In keeping with an active role of apoptosis in lumen formation, Survivin, a member of the inhibitors of apoptosis protein family (IAP) is expressed in the nucleus of epithelial cells bordering the forming lumina as the canals start to form (Jaskoll et al. 2001).

Programmed cell death can be triggered by different molecular pathways. The extrinsic pathway involves apoptotic signalling from outside the cell, mediated by members of the tumor necrosis factor receptor superfamily (Elmore 2007). The intrinsic pathway depends on endogenous factors to activate apoptosis and is stimulated by intracellular stress such as DNA damage, hypoxia, oncogene activation and deprivation of growth factors. The mitochondria is the central organelle of this pathway and releases several pro-apoptotic molecules into the cytoplasm (Elmore 2007; Taylor et al. 2008).

Given the specific functions of the SG ductal system to properly secrete and deliver saliva in the oral cavity, it is important to understand the mechanisms involved in SG formation. In other systems cell polarisation, apoptosis, anoikis and cord hollowing have been suggested to play a role in epithelial

tubulogenesis (Nedvetsky et al. 2014; Mailleux et al. 2007; Debnath et al. 2002; Andrew and Ewald 2010). Here we have concentrated on the role of apoptosis, analysing the temporal expression of apoptotic markers during early SG development, and using inhibition of apoptosis in culture, introducing a potential early role of caspase-dependent apoptosis in lumen formation starting prior to branching morphogenesis.

Material and Methods

Organ culture: Mouse tissue was collected from wild type (WT) CD1 animals housed in the Biological Services Unit in New Hunts House at King's College London, in approved non-SPF conditions. Mice were culled using schedule one culling methods, as approved by the Home Office and King's College London. Animal experiments conform to ARRIVE Guidelines. Embryonic SGs at stages E12.5 to E14.5 were dissected out for cultures and immunohistochemistry analysis (N=6 of each stage and group).

Pan-caspase inhibitor Z-VAD-FMK (G7231, Promega) was used for *in vitro* apoptotic inhibition at a range of concentrations (100, 150, 200 μ M) at E13.5 and E14.5 (N = 9) and at 100 μ M at E12.5 (N=18). Samples were placed on permeable membranes (BD) over culture medium (Advanced Dulbecco Modified Eagle Medium F12, Invitrogen; 1% GlutamaxTM, Invitrogen; 1% penicillin-streptomycin) at 37°C and 5% CO₂. Controls were cultured in DMSO at the same concentration. *In situ* CaspACE FITC-Z-VAD-FMK marker (G7461, Promega) was added to the culture medium in the last day for 3h. Cultured SGs were then fixed for 30min at room temperature (RT) and washed in PBS.

Lysotracker RED (DND-99, Invitrogen) was added in culture medium (5 μ M) of live SGs (N=6 of each stage) for 3h. After washing with fresh culture medium and PBT, samples were fixed in 4% paraformaldehyde for 30min at RT. Samples were permeabilised with PBT for 15min, followed by incubation in BS for 2h and DAPI (1:1000, Invitrogen) for 4h at RT. For immunohistochemistry/immunofluorescence and morphometric analysis see supplementary methods.

Results

Previous papers have concentrated on apoptosis during duct cavitation from E14.5 onwards, we therefore wanted to analyse whether apoptosis also had a role during earlier stages of gland development, when the main stalk of the gland is formed. Morphological analysis of early stages is shown in Fig. 1 (A, E, I), and the epithelial structures of forming SGs are highlighted with E-cadherin staining (Fig. 1B, F, J). The opening of the first luminal space appears from E13.5 (Fig. 1E, arrow). Specific apoptotic activity was confirmed using cleaved Caspase-3 and TUNEL at the same stages (Fig. 1C,G,K and 1D,H,L respectively; Suppl. Fig.1). Both markers were found in the centre of the main epithelial stalk at E12.5 where the lumen space is due to form (Fig. 1C,D). High levels of apoptotic cells were observed at E13.5 within the main duct and the oral epithelium (Fig. 1G, H) (Suppl. Fig. 1). Interestingly, sparse (Fig. 1K, arrow) or no positive cells (Fig. 1L, arrowheads) were observed in the more developed ducts at E14.5. At E12.5, subtle luminal gaps were observed within the main stalk (Fig. 1M, arrowheads), and at E13.5 they were clear within the duct associated with dying cells (Fig. 1O, arrowheads). In contrast the duct nearer the branching region had no yet initiated lumen formation. At these early stages, dying cells were found in the centre of the main epithelial stalk using Lysotracker staining, which labels lysozymes (Fig. 1N, P, arrows) (Fogel et al. 2012). Co-localisation of Lysotracker and cleaved caspase-3 expression was observed in the duct of the submandibular gland at E13.5 (Fig. 1Q to T). In the sublingual gland, which is delayed in development compared to the neighbouring submandibular (Redman and Ball 1978), cleaved caspase-3 positive cells were observed running along the forming duct, while Lysotracker was not yet expressed (Fig.1R), confirming that presence of cleaved caspase-3 is an early indicator of luminal space opening.

In order to confirm the presence of the forming lumen, Cytokeratin 7 (K7) was used to identify the presumptive luminal areas within the ducts as previously reported in developing SGs (Walker et al. 2008), and also in less differentiated cells of the ductal tree in the mammalian pancreas (Bowens 1998) (Fig. 2). At E12.5, K7 expression was observed in the central cells of the epithelial stalk connected to the single bud (Fig. 2A, arrows), while expression was completely absent in the bud itself (Fig. 2A, arrowhead). At E13.5, a stronger expression was observed in the luminal cells of the main duct, lining the developing gaps (Fig. 2B, arrow). Towards the canalisation stage at E14.5, the expression of K7 remained positive in the centre of the ductal structures where clear lumens were developing (Fig. 2C, arrows). During these stages K7 expression was also observed in the oral epithelium (OE), with expression increasing at later stage (E14.5) (Fig. 2D, arrow).

The role of apoptosis in the main stalk was analysed by *in vitro* culture to block apoptosis using a pan-caspase inhibitor at 100 μ M in the developing glands. Caspases are the main effectors and activators of both the intrinsic and extrinsic apoptotic pathways (Degterev et al. 2003; Taylor et al.

2008). SG explants at E12.5 were cultured for 72 hours. During this period the WT cultures developed from a bud-like thickening to a main bud that had started to cleft, attached to the oral epithelium by a narrow stalk. In contrast the stalk region failed to narrow in the caspase treated explants (Fig. 3A, arrows). The diameter of the main primary stalk of each gland was measured in both experimental and control groups at the start and during culture, resulting in statistically significant difference from 24h (Fig. 3B), ($p < 0.05$) ($N = 18$). E12.5 cultures developed much slower than those without DMSO, the carrier used, indicating that this early stage is sensitive to even fairly low levels of this vehicle. In agreement with this higher concentration of inhibitor, and therefore carrier, led to arrest of gland development (data not shown). We therefore performed a similar functional experiment using E13.5 and E14.5 SGs to assess the effects of pan-caspase inhibition on duct development at later stages. For this we used the concentrations of 100 μ M plus 150 μ M and 200 μ M. At E13.5 the addition of 150 μ M and 200 μ M inhibitor led to complete loss of apoptotic cells, as determined by activated caspase-3 staining, after 20 hours in culture, while at 100 μ M a few positive cells were observed indicating some cells had escaped inhibition at this concentration at this stage (Fig. 4A, arrows) ($N = 3$ each concentration). At E13.5 addition of Z-VAD-FMK inhibitor resulted in impaired stalk narrowing in a dose-dependent manner (Fig. 4B). At 150 μ M a statistically significant failure in narrowing was observed after 72 hours in culture (Fig. 4C). The wide morphology of the ductal structures after treatment agrees with the data from E12.5, and suggesting that caspase-dependent apoptosis plays a role in the narrowing of the ducts prior to lumen formation. At E13.5, branching was also impaired showing reduction in the number of buds when cultured at higher concentrations of inhibitor (Fig. 4D, Fig. 5A-F). In contrast, E14.5 SG cultures showed no abnormality within all groups (Suppl. Fig. 2), agreeing with the fact that only low levels of apoptosis are evident at this stage (see Fig.1).

In order to label cells that had been blocked by the inhibitor, plus cells that were undergoing cell death just before fixation, the apoptotic inhibitor marker FITC Z-VAD-FMK was added to E13.5 cultures at 72hours ($N = 9$) (5A'-F'). No labelling was observed in the SG epithelium of the control cultures, indicating low levels of apoptosis at this stage of development (Fig. 5A',C',E'), agreeing with the findings from Fig. 1G,J. A few positive cells were evident in the mesenchyme, increasing with higher concentration of DMSO, indicating some cell death in the mesenchyme at the end of the culture period. This again highlights the toxicity of DMSO for developing SGs and the need to be careful when analysing results when using higher concentrations. In the inhibitor treated cultures large numbers of positive cells were concentrated at the centre of the main ducts, corresponding to the pattern of apoptosis observed in glands *in vitro* at the start of culture (Fig. 5B',D',F' compared to Fig 1). In addition to the effect on width of the duct, lumen formation also appeared abnormal. In the controls at 100 μ M and 150 μ M clear, wide, cell free lumens formed in the central stalk (Fig. 5A,A',B,B'). In contrast, this region in the caspase inhibitor cultures was filled with FITC labelled cells (Fig. 5D). In

section the lack of lumen was clear in the inhibitor treated cultures (Suppl. Fig. 1). In addition to defects in duct morphogenesis E13.5 samples also showed a dose dependent defect in branching morphogenesis (Fig. 5). The reduced branching was not expected as very few apoptotic cells were associated with this part of the gland at these stages, however the FITC marker also showed high levels of inhibitor binding in the mesenchyme, particularly at high concentrations (Fig. 5D', F') suggesting that the inhibitor may also have impacted on normal development of the mesenchyme, therefore indirectly affecting branching morphogenesis.

Discussion

Apoptosis has been recognized as one of the most important mechanisms involved in developmental dynamics (Flusberg and Sorger 2015) with the large caspases family being key regulators of the main programmed cell death pathways (Degterev et al. 2003; Kuranaga 2011; Hengartner 2000; Taylor et al. 2008). Branching morphogenesis regulates the formation of distinct organs like lungs, kidney, mammary and salivary glands, which can perform essential physiological functions, such as exchanging gases or production of secretions (Ochoa-Espinosa and Affolter 2012; Patel et al. 2006; Patel and Hoffman 2014; Larsen et al. 2010). Studies on epithelial tube morphogenesis have highlighted the involvement of distinct methods for lumen formation depending on the type of organ involved (Andrew and Ewald 2010; Chung and Andrew 2008; Lubarsky and Krasnow 2003). In SGs, it has been suggested that the central cells of the epithelial stalks undergo apoptosis in a process known as cavitation, clearing the luminal space during embryonic SG development (Wells and Patel 2010; Tucker 2007). Corroborating with these previous reports, our findings illustrate the presence of dying cells in the centre of the presumptive ducts as shown by Lysotracker staining, activated caspase-3 and TUNEL. Temporal expression of apoptotic cells in duct formation however diverged from previous studies. To date, it has been reported that mouse SG lumen spaces start opening only from stages E14.5 or E15.5, with apoptotic cells and cleaved caspase-3 observed from E14.5 (Jaskoll and Melnick 1999; Melnick et al. 2001). It is therefore assumed that SGs are comprised of solid and non-luminised cords before this time point (Jaskoll et al. 2001; Larsen et al. 2006; Tucker 2007). In this study, apoptotic cells were detected at earlier stages, from E12.5, suggesting an important early role for apoptosis in the formation of the lumen of the central SG ducts, and the central duct was shown to open at early stages of development before the rest of the gland had undergone branching morphogenesis. This finding was further supported by the expression of K7 in the centre of the main stalk of developing SGs from E12.5 (Walker et al. 2008). Such discrepancy could be due to the fact that earlier stages were not studied in details or that different methods for detecting apoptosis were employed. Melnick and Jaskoll reported the presence of apoptotic cells only from E14.5 using the Apostain assay, which identifies DNA after

caspase cleavage and before DNA fragmentation (Jaskoll and Melnick 1999). Comparison between TUNEL and Apostain assays have reported good reliability of both techniques although their outcomes showed slightly variations in the timing and amount of expression (Prochazkova et al. 2003). Our results revealed scarce/occasional apoptotic cells (cleaved caspase-3 and TUNEL positives) at later stages E14.5 and E15.5, and no overt defect in gland development in cultures treated with inhibitor from E14.5, indicating a more minor role for apoptosis at these later time points.

Functional experiments were performed to assess the effects of suppressing apoptotic activity in SG development through *in vitro* pan-caspase inhibition. The Z-VAD-FMK inhibitor binds irreversibly to active caspases, and from the lowest concentration of 100 μ M it was able to compromise SG development at early stages. In previous studies in epithelial cell cultures, apoptotic inhibition was sufficient to avoid lumen formation when cell polarisation was not enhanced (Martín-Belmonte et al. 2008). However when cell polarisation was stimulated in prostate epithelial models, inhibition of apoptosis only delayed lumen formation (Pearson et al. 2009). Studies with mammary cell cultures have also shown similar results by inhibiting apoptosis, correlating apoptosis with generation and maintenance of hollow lumen spaces (Debnath et al. 2002).

Defects in branching and duct formation due to pan-caspase inhibition revealed an important function of apoptosis during SG development. However it is worth bearing in mind that caspase inhibition can trigger the activation of non-caspase dependent cell death pathways, such as necrosis and autophagy, which could compensate for the absence of apoptosis (Vandenabeele et al. 2006). Additionally, it has been reported that caspase functions are not exclusively apoptotic, being also correlated to dynamic cell processes such as cell-fate determination, compensatory proliferation of neighboring cells, and actin cytoskeleton reorganization, in a non-apoptotic context during mouse development (Kuranaga 2011; Wang and Lenardo 2000). Caspase inhibition can therefore potentially affect other essential mechanisms during embryogenesis, which might have resulted in the severe phenotypes observed in our *in vitro* cultures not only in duct formation but also in epithelial branching. The importance of apoptosis during organ development has recently been correlated to effects on proliferation, movement and shape of neighbouring cells (Pérez-Garijo and Steller 2015), suggesting an additional function for those apoptotic cells during early SG development.

Studies analysing epithelial cell interactions during SG development have shown links between control of lumen formation and cell polarisation (Cutler and Mooradian 1987; Andrew and Ewald 2010), supported more recently by genetic links between the two processes (Nedvetsky et al. 2014). Neuronal-derived vasoactive intestinal peptide (VIP) was found as an important regulator of key steps during epithelial tubulogenesis in SG development, where exogenous VIP stimulated the formation of a single contiguous lumen space, as marked by apical aPKC and subapical GM130, from E13. Moreover

it was reported that VIP-induced lumen formation did not require apoptosis, showing normal lumen formation in SGs cultured with pan-caspase inhibitor. In this experiment the inhibitor was used at 50 μ M, whereas we saw only partial inhibition using a higher concentration of 100 μ M, the lack of phenotype in the Nedvetsky paper could therefore be due to the low dose of Z-VAD-FMK used. Normal lumens were also observed in Bax knockout mice, however they were only analysed at E17. In this case, it is possible that Bax-dependent lumen formation was compensated for by other cell death mechanisms or analogous molecules. This protein is only required for intrinsic apoptosis and it shares similar pro-apoptotic function with another apoptotic protein Bak (Karbowksi et al. 2006; Lindsten et al. 2000). Compensation by other cell death pathways is a common mechanism observed in other areas where cell death has been investigated, for example in the limb (Miura 2012; Suzanne and Steller 2013).

Another cell mechanism named anoikis has also been indicated as an important process during epithelial tube formation, as seen in mammary gland morphogenesis (Debnath et al. 2002; Mailleux et al. 2007). This is based on the loss of cell anchorage, which disturbs cell-cell and cell-extracellular matrix adhesion interactions, playing important role in the physiological induction of apoptosis during development and pathogenesis, mainly regarding metastatic transformation (Grossmann 2002). Corroborating with this hypothesis, in our SG explant cultures the apoptotic inhibition marker FITC-Z-VAD-FMK indicated the presence of many unorganised cells remaining in the centre of the presumptive duct, not showing a clear lumen space and being detached from the polarised epithelial cells lining the cavity. Given that anoikis is also potentially responsible for triggering apoptosis during lumen space opening, cavitation could actually represent a special case of cord hollowing, which requires cell polarization and the extra step of clearing the lumen (Andrew and Ewald 2010).

In conclusion, initial lumen formation in the SG main stalk seems to occur by an apoptosis driven mechanism. The decreasing expression of apoptotic cells through SG development either in ducts or acini suggests that further lumen formation and maintenance of the subsequent ducts possibly use other mechanisms. Cell polarisation, anoikis and non-apoptotic cell death pathways may be essential to these developmental processes. It will therefore be important to further explore these ideas in order to establish a definite causal relationship between lumen formation, lumen maintenance and cell death.

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Figure legends

Figure 1: Cell death in early SG development

(A, B, C, D) E12.5. (E, F, G, H) E13.5. (I, J, K, L) E14.5. (M, N, O, P) Main duct of submandibular gland. (Q, R, S) E13.5 SG.

(A, E, I) Histology sagittal sections of E12.5 to E14.5 SG (B, F, J) E-cadherin expression in developing SG epithelium. (C, G, K, S) Activated caspase-3. (D, H, L) TUNEL. (M, O) DAPI staining with characteristic apoptotic condensed nuclei visible in the central lumen area (arrowheads). (N, P, R) Lysotracker pseudocoloured green from red. At E12.5 the first signs of apoptosis are visible as shown by all three markers (C, D, N, arrows), with higher number at E13.5 as the gland starts to branch (G, H, P, arrows). At E14.5 scarce or no positive cells are present in the lumen spaces within the ducts (K, L). Arrows indicate positive cells, arrowheads indicate forming lumens. OE = Oral epithelium. (Q, R, S, T) Comparison between Lysotracker and cleaved caspase-3 expression (Q, R, arrows) localisation in the same E13.5 SG sample. Cleaved Caspase-3 positive cells are present mainly within the duct of the sublingual gland, located to the left of the larger submandibular gland. Lysotracker is mainly visualised in submandibular ductal cells. High magnification of the main forming duct in the submandibular gland showing both Lysotracker and cleaved caspase-3 expression is shown in T. Scale bar = 100µm.

Figure 2: Presumptive lumen formation as labelled by Cytokeratin (K) 7

(A) K7 positive cells are localised at the centre of the initial solid stalk at E12.5 (arrows). (B) K7 remains expressed at E13.5 restricted to the presumptive luminal area (arrow). (C) At E14.5 K7 is more evident within the lumen space of SG ducts. (D) K7 is also expressed in the oral epithelium. Asterix marks submandibular gland. Scale bar = 100µm.

Figure 3: Early inhibition of caspases leads to defects in duct morphology

(A) E12.5 SG explants cultured for 72h. A defect in duct morphology is evident between the control (DMSO) and the experimental (ZVAD) group after 48h and 72h of culture. Arrows indicate the ducts at 72hrs. Scale bar = 500µm.

(B) Paired student t-test statistical analysis of the duct diameter of both groups in E12.5 SG cultures. No difference in duct dimensions was observed prior to culture at 0h, compared to statistically significantly narrowing of the ducts in control cultures compared to treated cultures after 24, 48 and 72 hours.

N=18 for E12.5 and N=9 for E13.5 and E14.5 glands. * $p = 0.02$; ** $p = 0.008$; *** $p = 0.0006$. Error bars represent one standard error of the mean.

Figure 4: Loss of activated caspase-3 leads to a reduction in duct narrowing and branching at the pseudoglandular stage

(A) Expression of cleaved caspase-3 within developing submandibular ducts of treated and non-treated E13.5 SG cultures after 20h (arrows). High levels of cleaved caspase-3 (red) positive cells were found within developing lumen space of all controls (left hand side column). From 150 μ M of Z-VAD-FMK, cleaved caspase-3 was completely absent, with a few labelled cells present at 100 μ M (right hand column). Scale bar = 100 μ m.

(B) Unpaired t-test comparing the mean duct diameter increase (Day3-Day 0) of E13.5 SGs cultured for 72h for the three different Z-VAD-FMK concentrations. N = 9, * $p = 0.05$.

(C) Paired student t-test statistical analysis of the final duct diameter of both groups in E13.5 SG cultures. No difference in duct dimensions were observed prior to culture at 0h, compared to statistically significantly narrowing of the ducts in control cultures compared to treated cultures after 24, 48 and 72 hours. N = 18, * $p = 0.03$.

(D) Mean Spooner ratio for treated (Z-VAD-FMK) and non-treated groups (DMSO) after 48h. Paired student t-test show a significant decrease in branching using 150 and 200 μ M of inhibitor. N = 9, * $p = 0.01$, ** $p = 0.006$.

Figure 5: Inhibition of caspases at E13.5 leads to loss of lumen formation in the main stalk

(A-F) Explants cultured from E13.5 for 72hours, with or without Z-VAD-FMK inhibitor. FITC-Z-VAD-FMK added to all cultures prior to fixation to label cells that are currently undergoing apoptosis (control and treated group, A'-F'), and caspase positive cells that have previously been inhibited from undergoing apoptosis (treated group).

(A, C, E) Morphological images of control explants (DMSO treated). Arrowheads indicate areas of branching morphogenesis. **(A', C', E')** Confocal images of the same control group highlighting absence of apoptotic inhibition inside the main ducts (arrows) when treated with FITC-Z-VAD-FMK before fixation. **(E)** Some apoptosis (green label) is evident in the mesenchyme at high levels of DMSO. **(B, D, F)** Pan-caspase inhibition at a range of concentrations, demonstrating defects in branching morphogenesis (arrowheads) and expansion of the duct diameter (arrows) in the absence of caspases.

(B', D', F') Confocal images of the same treated group highlighting presence of apoptotic inhibition (green labelled cells) inside the main ducts (arrows). The main ducts are filled with cells rather than cell-free lumen, as seen in controls. High levels of apoptosis (green label) are observed in the mesenchyme. Scale bar = 500µm.

Supplementary Figure 1: Apoptosis in early salivary gland development and its role in lumen formation

(A) TUNEL positive cells in the forming luminal area at E12.5 (arrow). **(B)** E13.5 SG sections with TUNEL positive cells in the ductal region (arrow). **(C,D)** Histological sections of E13.5 explants after 3 days of culture in DMSO (C – control group) and in pan-caspase inhibitor Z-VAD-FMK (D). Arrows exhibit the presence of well-formed luminal spaces in C, while wide non-luminised stalks are observed in the absence of caspase activation (D).

Supplementary Figure 2: Apoptotic inhibition at E14.5 does not affect branching and lumen formation

(A-F) Explant cultures of E14.5 SG after 48h of treatment with and without Z-VAD-FMK at different concentrations. **(A'- F')** Respective morphology of the explants with DAPI staining. Lumen space is formed normally at all concentrations of inhibitor at this stage of development **(B', D', F', arrows)**. Scale bar = 500µm.













